THE DEPENDENCE OF CELL-FREE PROTEIN SYNTHESIS IN *E. COLI* UPON RNA PREPARED FROM RIBOSOMES

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A stable cell-free system has been obtained from *E. coli* which incorporates C^{14}-valine into protein at a rapid rate. This system is of particular interest since certain parameters resemble protein synthesis in intact cells (1). The purpose of this communication is to describe a novel characteristic of the system; that is, a requirement for high molecular weight ribosomal RNA, needed even in the presence of soluble RNA and ribosomes.

Washed *E. coli* W3100 cells harvested in early log phase were disrupted by grinding with alumina and enzymes were extracted with 2.5 volumes of buffer containing 0.01 M Tris, pH 7.8, 0.01 M Mg acetate, 0.06 M KCl, 0.006 M mercaptoethanol (standard buffer). The extract was centrifuged at 20,000 x g for 20 minutes; supernatant fluid was decanted and 3 µg DNAase per ml was added to reduce its viscosity. The supernatant fluid was centrifuged again at 20,000 x g for 20 minutes and the precipitate was discarded. The remaining solution was centrifuged again at 30,000 x g for 30 minutes. This supernatant layer (S-30) was recentrifuged at 105,000 x g for 2 hours to sediment the ribosomes. The supernatant solution (S-100) was aspirated and the ribosomes were washed in standard buffer and centrifugation as before (W-Rib). Fractions S-30, S-100, and W-Rib were dialyzed against standard buffer overnight at 5°, and were stored at -15°.

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In most cases fresh S-30 was incubated for 40 minutes at 35° under the conditions described in Table 1, except that C^{12}-L-valine was present in place of U-C^{14}-L-valine. After incubation the reaction mixture was dialyzed 10 hours at 5° against standard buffer which was changed once. The enzyme was stored at -15°. (Incubated-S-30).

RNA fractions were prepared by three phenol extractions at 24° of fresh washed ribosomes; 1, 1/2 and 1/2 volumes of H_2O-saturated phenol were used which were concentrated by precipitation with 2 volumes of ethanol and exhaustively dialyzed against standard buffer minus mercaptoethanol.

In Fig. 1 amino acid incorporation into protein is presented as a function of time. In the absence of E. coli ribosomal RNA, amino acid incorporation ceases after 30 minutes. Saturating concentrations of E. coli soluble RNA were present in every reaction mixture. Increasing the concentration of soluble RNA 3-fold did not increase the amino acid incorporation into protein. However, when ribosomal RNA was added, a marked increase in amino acid incorporation occurred. At low concentrations of ribosomal RNA, maximum amino acid incorporation into protein was proportional to the amount of ribosomal RNA added, suggesting a stoichiometric rather than catalytic action of ribosomal RNA. In contrast, soluble RNA has recently been shown to act in a catalytic fashion (3).

In Table 1 are presented the characteristics of C^{14}-L-valine incorporation into protein due to the addition of ribosomal RNA. Amino acid incorporation was dependent upon the addition of ATP and an ATP-generating system and was completely inhibited by the addition of RNAase but not DNAase. Heating ribosomal RNA to 100° for 10 minutes did not destroy its activity. Amino acid incorporation was inhibited by 0.15 μmoles/ml chloramphenicol and 0.20 μmoles/ml puromycin. The addition of 20 different L-amino acids markedly stimulated the incorporation of C^{14}-valine into protein. Therefore, amino acid incorporation due to the addition of ribosomal RNA had many characteristics expected of protein synthesis.

In all experiments amino acid incorporation was dependent upon the addition of both ribosomes and 105,000 x g supernatant fluid, indicating that contamination

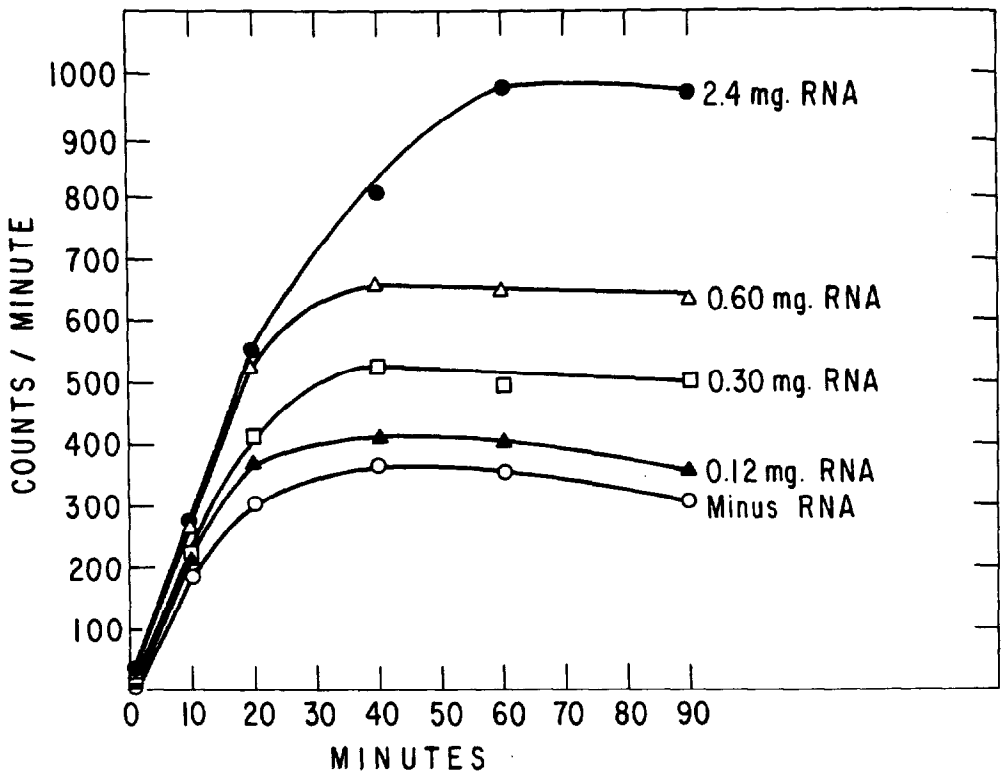

Fig. 1 - Dependence of C^{14}-L-valine incorporation into protein upon ribosomal RNA. The reaction mixture is presented in Table 1. Each reaction mixture contained 0.98 mg of E. coli soluble RNA (saturating concentration) and 4.4 mg of Incubated-S-30 protein.

of enzyme preparations by intact cells was highly unlikely. Ribosomal RNA had no effect upon amino acid incorporation into protein in the absence of ribosomes and the RNA could not be replaced by the addition of equivalent concentrations of polyanions such as polyadenylic acid, highly polymerized salmon sperm DNA, or a high molecular weight polymer of glucose carboxylic acid. Pretreatment of ribosomal RNA with trypsin did not affect its biological activity. However, treatment of the ribosomal RNA with RNAase (followed by removal of protein), or alkali, completely destroyed its biologic activity. Thus, all of the activity appeared to be associated with RNA.

Preliminary attempts at fractionation of the ribosomal RNA have been performed by means of density-gradient centrifugation in sucrose (4). Biological activity of

TABLE 1

Characteristics of C^{14}-L-Valine Incorporation into Protein

Expt. No.		Counts/minute/mg protein
1	- Ribosomal RNA	42
	+ " "	204
	+ " " + 0.15 μmole Chloramphenicol	58
	+ " " + 0.20 μmole Puromycin	7
	+ " " Zero time	8
2	- Ribosomal RNA	35
	+ " "	101
	+ " " - ATP, PEP, PEP kinase	7
	+ " " + 10 μg RNAase	6
	+ " " + 10 μg DNAase	110
	+ Boiled Ribosomal RNA	127
	+ Ribosomal RNA, Zero time	8
3	- Ribosomal RNA	34
	- " " - 20 L-amino acids	21
	+ " "	99
	+ " " - 20 L-amino acids	52

The reaction mixtures contained the following in μmole/ml: 100, Tris(hydroxymethyl)aminomethane, pH 7.8; 10 Mg acetate; 50 KCl; 6.0 mercaptoethanol; 1.0 ATP; 5.0 phosphoenolpyruvate, K salt; 20 μg phosphoenolpyruvate kinase, crystalline; 0.05 each of 20 L-amino acids minus valine; 0.03 each of GTP, CTP and UTP; 0.019 C^{14}-L-valine (~ 70,000 counts); 3.1 mg E. coli ribosomal RNA where indicated, and 1.0 mg E. coli soluble RNA (saturating concentrations); 3.2, 3.2 and 1.4 mg of Incubated-S-30 protein were present in Expts. 1, 2, and 3, respectively. In addition 4.4 mg protein of W-Rib were added in Expt. 3. Total volume was 1.0 ml. Samples were incubated at 35° for 20 minutes, were deproteinized with 10% trichloracetic acid and the precipitates were washed and counted by the method of Siekevitz (2).

the RNA did not follow absorbancy at 260 mμ, instead the activity was concentrated around a fraction sedimenting about three times as fast as soluble RNA. Investigation of ribosomal RNA preparations with the Spinco Model E ultracentrifuge showed two major peaks with S_{20W} values of about 23, 16 and a minor one at 4.4, corresponding to those described by Aronson and McCarthy (5). Addition of RNAase, but not trypsin, destroyed these peaks. It is possible that part or all of the ribosomal RNA used in our study corresponds to template or messenger RNA. The function of the ribosomal RNA in our system and its further purification are being studied now.

In summary, a stable, cell-free system has been obtained from E. coli in which

the incorporation of amino acids into protein was dependent on the addition of heat-stable ribosomal RNA preparations. Soluble RNA could not replace ribosomal RNA fractions. In addition, the amino acid incorporation required both ribosomes and 105,000 x g supernatant solution. The kinetics of the incorporation suggested stoichiometric rather than catalytic activity of ribosomal RNA. The ribosomal RNA-dependent amino acid incorporation also required ATP and an ATP-generating system, was stimulated by a complete mixture of L-amino acids, and was markedly inhibited by puromycin, chloramphenicol and RNAase.

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